Vitamin D₂ impairs utilization of vitamin D₃ in high-yielding dairy cows in a cross-over supplementation regimen

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ABSTRACT

Vitamin D exists in 2 forms that are important regarding vitamin D status and supply in cattle: vitamin D₂ (D₂) and vitamin D₃ (D₃). To become physiologically active, both D₂ and D₃ must undergo 25-hydroxylation in the liver. The resulting 25-hydroxyvitamin D₂ [25(OH)D₂] and 25-hydroxyvitamin D₃ [25(OH)D₃] are measured as indicators of the physiological vitamin D status of cattle. The study used 14 Danish Holstein cows housed without access to sunlight. The cows were orally administered 250 mg (1.0×10^7 IU) of D₂ and D₃ in a cross-over design with 2 treatment groups and 2 study periods, rendering 4 treatments when carryover effects were taken into account: D₂ given first, D₂ given last after D₃, D₃ given first, and D₃ given last after D₂. Two weeks elapsed between the treatment in the first study period and the treatment in the second study period. Blood samples were collected 0, 3, 6, 14, 17, 20, 23, 26, 40, 48, 70, 94, 166, and 214 h after providing the oral bolus of vitamin to the cows. Comparisons between plasma levels of the metabolites D₂, D₃, 25(OH)D₂, and 25(OH)D₃ over time were made by comparing areas under the plasma concentration curves. Oral administration of D₃ increased plasma D₃ (182.6 ± 17.1 ng/mL; mean $\pm$ SEM) and 25(OH)D₃ (103.5 ± 10.0 ng/mL) more efficiently than oral administration of D₂ increased plasma D₂ (49.1 ± 32.6 ng/mL) and 25(OH)D₂ (27.9 ± 2.1 ng/mL). The D₃ given after an oral dose of D₂ was less efficient for increasing plasma concentrations of 25(OH)D₃ (61.2 ± 12.0 ng/mL) compared with D₃ given without previous D₂ administration (103.5 ± 10.0 ng/mL), whereas the plasma concentrations of D₃ itself were the same when given first (182.6 ± 17.1 ng/mL) as when given after D₂ (200.0 ± 123.9 ng/mL). The same occurred for plasma concentrations of D₂ metabolites both if D₂ was given first (49.1 ± 32.6 ng/mL) and after D₃ (54.7 ± 7.7 ng/mL). In conclusion, D₃ given after D₂ is less efficient at increasing the plasma

status of 25(OH)D₃ than D₃ given without previous D₂ administration.

Key words: vitamin D₂, vitamin D₃, competition and discrimination, impaired utilization

INTRODUCTION

Vitamin D exists in 2 forms that are important regarding the vitamin D status and supply to cattle: vitamin D₂ (D₂), which is produced by fungi growing on plant material used as roughage for cattle (Richardson and Logendra, 1997), and vitamin D₃ (D₃), which is either produced endogenously in the skin of the animals during exposure to sunlight (Hymøller and Jensen, 2010a) or given in the feed as synthetic additives. To become physiologically active, both D₂ and D₃ must undergo hydroxylation in the liver to produce 25-hydroxyvitamin D₂ [25(OH)D₂] and 25-hydroxyvitamin D₃ [25(OH)D₃], respectively, which are measured in plasma as indicators of physiological vitamin D status.

The process of absorption of D₂ and D₃ from the gastrointestinal tract is not understood in detail; however, uptake of dietary D₃ in rats occurred by passive diffusion with the fat fraction of the feed in in vitro studies with everted small intestine and in in vivo studies with rats fitted with intestinal loops (Hollander, 1976; Hollander et al., 1978). It was assumed that D₂ followed a route similar to that of D₃ into circulation (Heymann, 1937).

Hymøller and Jensen (2010b) showed that even though orally administered D₂ and D₃ were stable in the rumen of high-yielding dairy cows and they entered the small intestine for absorption in similar amounts, D₂ and its liver-derived metabolite 25(OH)D₂ still circulated in plasma at lower concentrations than D₃ and its liver-derived metabolite, 25(OH)D₃, after dairy cows simultaneously ingested 250 mg of D₂ and D₃. Horst et al. (1982) found similar results in pigs and chickens. This difference in plasma concentrations is believed to be caused by more rapid metabolism and clearance of D₂ from plasma than D₃ (Armas et al., 2004), probably due to a lower affinity of the plasma-based vitamin D binding protein (DBP) for D₂ than D₃ or a higher

Received December 20, 2010.

Accepted March 14, 2011.

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affinity of the liver-based vitamin D-25-hydroxylase enzyme for D₃ than for D₂ (Horst et al., 1982; DeLuca et al., 1988; Armas et al., 2004).

A possible adverse effect of D₂ supplementation on the plasma status of D₃ has been reported in humans (Tjellesen et al., 1986; Armas et al., 2004), a phenomenon that should be further investigated in cattle due to its possible effect on the utilization of D₃ supplements when D₂ is present in roughage used for cattle feeding. For instance, Hidioglu et al. (1980) found that hay-fed heifers showed lower increases in plasma concentrations of total 25-hydroxyvitamin D [25(OH)D; 25(OH)D₂ and 25(OH)D₃ combined] compared with silage-fed heifers after i.m. injection of 25 mg (1.0×10^6 IU) of D₃, which could be caused by an adverse effect of a higher D₂ content in hay than in silage (Keener, 1954).

The aim was to identify whether D₂ supplementation before D₃ supplementation had adverse carryover effects on the physiological effect of D₃ supplementation in dairy cattle due to its possible effect on the utilization of D₃ supplements when D₂ is present in roughage used for cattle feeding.

MATERIALS AND METHODS

Animals

Fourteen Danish Holstein cows were assigned to 2 treatment groups balanced according to parity (first, second, and later) and milk yield (kg/d). The average milk yield was 32.4 ± 2.3 kg/d (mean $\pm$ SEM) at the beginning of the study and 31.9 ± 2.1 kg/d at the end of the study, 24 d later. The study complied with the Danish Ministry of Justice Law No. 726 (September 9, 1993) concerning experiments with animals and care of experimental animals.

Housing and Management

Cows were housed in tie stalls and fed ad libitum a TMR containing corn silage (30% of DM), clover grass silage (27% of DM), rapeseed cake (15% of DM), alfalfa silage (8% of DM), rolled barley (6% of DM), soybean meal (6% of DM), sugar beet molasses (4% of DM), and dried sugar beet pulp (4% of DM). The TMR was fed once daily at 0900 h and milking was carried out twice daily at 0600 and 1700 h. Use of commercial vitamin and mineral mixtures that contained D₃ in the TMR ended 28 d before the study and the cows were denied access to sunlight during the same period. The latter prevented interference from endogenous D₃ produced in the skin during exposure to sunlight (Hymøller and Jensen, 2010a).

Treatments

Two vitamin D metabolites, D₂ and D₃ (>98% pure), were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). A total of 250 mg (1.0×10^7 IU) of either D₂ or D₃ was weighed, mixed with soy protein and sugar beet molasses, and wrapped in a single layer of tissue paper to form a pill suitable for oral administration to the cows with a bolus gun. The amount of 250 mg of D₂ or D₃ was chosen as suitable for obtaining a high plasma response and saturation of the liver-based 25-hydroxylase enzymes based on previous research in the field (Hymøller and Jensen, 2010b).

The vitamin D metabolites were administered in a cross-over design with 2 treatment groups (group 1 and group 2) and 2 study periods (period 1 and period 2). Each study period lasted only 2 wk after the bolus was given to allow a carryover effect from period 1 to period 2. This gave 4 vitamin D treatments: D₂ first (**D2F**), D₂ last after D₃ (**D2L**), D₃ first (**D3F**), and D₃ last after D₂ (**D3L**). The same designations were used when comparing plasma results of D₂ and D₃ in the different study groups and treatments. The corresponding plasma 25(OH)D₂ and 25(OH)D₃ groups were designated **25D2F**, **25D2L**, **25D3F**, and **25D3L**, respectively (Table 1).

Samples

Blood samples were collected from the jugular vein in EDTA-coated Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ) 0, 3, 6, 14, 17, 20, 23, 26, 40, 48, 70, 94, 166, and 214 h after giving 1 oral bolus of either D₂ or D₃ to the cows. The samples were centrifuged for 10 min at $1,500 \times g$ and the plasma was

Table 1. Cross-over design of the 4 treatments with oral doses of 250 mg (1.0×10^7 IU) of vitamin D₂ or vitamin D₃, respectively

Period	Treatment group ¹	
	1	2
1	250 mg of vitamin D ₂ (D2F and 25D2F)	250 mg of vitamin D ₃ (D3F and 25D3F)
2	250 mg of vitamin D ₃ (D3L and 25D3L)	250 mg of vitamin D ₂ (D2L and 25D2L)

¹Plasma response of different vitamin D metabolites after the 4 treatments in the crossover design: D2F = plasma vitamin D₂, when vitamin D₂ was given first; D2L = plasma vitamin D₂, when vitamin D₂ was given last after vitamin D₃; D3F = plasma vitamin D₃, when vitamin D₃ was given first; D3L = plasma vitamin D₃, when vitamin D₃ was given last after vitamin D₂; 25D2F = plasma 25-hydroxyvitamin D₂, when vitamin D₂ was given first; 25D2L = plasma 25-hydroxyvitamin D₂, when vitamin D₂ was given last after vitamin D₃; 25D3F = plasma 25-hydroxyvitamin D₃, when vitamin D₃ was given first; 25D3L = plasma 25-hydroxyvitamin D₃, when vitamin D₃ was given last after vitamin D₂.

Table 2. Initial plasma concentrations (ng/mL; $\pm$ SEM) of vitamin D metabolites before each study period

Metabolite	Treatment ¹			
	D2F	D2L	D3F	D3L
25-Hydroxyvitamin D ₂	5.7 $\pm$ 1.2	6.7 $\pm$ 0.5	6.5 $\pm$ 0.8	28.6 $\pm$ 2.6
25-Hydroxyvitamin D ₃	37.2 $\pm$ 2.3	87.5 $\pm$ 5.8	35.6 $\pm$ 3.9	27.0 $\pm$ 3.0
Vitamin D ₂	0.2 $\pm$ 0.1	1.6 $\pm$ 3.4	0.1 $\pm$ 0.2	4.5 $\pm$ 3.0
Vitamin D ₃	1.9 $\pm$ 2.3	13.0 $\pm$ 1.5	0.9 $\pm$ 2.2	3.7 $\pm$ 2.4

¹D2F = plasma vitamin D₂, when vitamin D₂ was given first; D2L = plasma vitamin D₂, when vitamin D₂ was given last after vitamin D₃; D3F = plasma vitamin D₃, when vitamin D₃ was given first; D3L = plasma vitamin D₃, when vitamin D₃ was given last after vitamin D₂.

transferred to storage tubes and kept at -18°C until analysis.

Chemical Analysis

Plasma samples were analyzed for content of D₂ and D₃, together with their liver-derived metabolites 25(OH)D₂ and 25(OH)D₃, in the laboratories at Aarhus University, Faculty of Agricultural Sciences (Tjele, Denmark). After saponification and liquid-liquid extraction, separation was carried out by reverse-phase gradient HPLC on a C₃₀ column from YMC (Dinslagen, Germany) and UV detection at 265 nm. The metabolite 1 α -hydroxyvitamin D₃ [1 α (OH)D₃] from Sigma Aldrich Chemie GmbH (Steinheim, Germany) was used as internal standard for quantification. The method is described in detail by Hymøller and Jensen (2011).

Statistical Analysis

To compare the vitamin D status of the cows under different treatment regimens, definite integrals of D₂, D₃, 25(OH)D₂, and 25(OH)D₃ concentration curves over time from individual cows were approximated by numerical integration, applying the trapezium rule:

$$\int_{t=0 \text{ h}}^{t=214 \text{ h}} f(t)dt \approx \sum_{t=0 \text{ h}}^{t=214 \text{ h}} \left[t - (t-1) \right] x \frac{f(t-1) + f(t)}{2},$$

where $t = 0, 3, 6, 14, 17, 20, 23, 26, 40, 48, 70, 94, 166$, and 214 h. Results were compared by Student's 2-sample, 2-tailed, homoscedastic t -test.

RESULTS

Initial plasma concentrations of vitamin D metabolites at the beginning of study period 1 and 2, respectively, are in Table 2.

D₂ and D₃ in Plasma

The D2F and D2L plasma concentrations curves are in Figure 1. The area under the D2F plasma concentra-

tion curve was smaller than the area under the D2L plasma concentration curve with a curve area ratio of 0.81 ($P \leq 0.05$), whereas the areas under the D3F and D3L plasma concentration curves (Figure 2) were the same. The areas under the D3F and D3L plasma concentration curves were larger than the areas under the D2F and D2L plasma concentration curves, with curve area ratios of 4.96 and 3.70, respectively ($P \leq 0.001$).

25(OH)D₂ and 25(OH)D₃ in Plasma

The area under the 25D3F plasma concentration curve was larger than the area under the 25D2F plasma concentration curve, with a curve area ratio of 3.93 ($P \leq 0.001$) despite correcting for initial plasma concentrations of 25(OH)D₂ and 25(OH)D₃, respectively (Figure 3). Areas were corrected for initial plasma concentrations of 25(OH)D₂ and 25(OH)D₃ because different concentrations of these metabolites were found in plasma at the beginning of each study period (Table 2). The same occurred when comparing areas under the 25D2L (Figure 4) and 25D3L (Figure 5) plasma concentration curves, with a curve area ratio of 3.50 ($P \leq 0.05$).

The area under the 25D2L plasma concentration curve was reduced compared with the area under the

Figure 1. Vitamin D₂ concentration in plasma (ng/mL; $\pm$ SEM) after an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₂ given before (D2F) or after (D2L) an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₃.

Figure 2. Vitamin D₃ concentration in plasma (ng/mL; $\pm$ SEM) after an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₃ given before (D3F) or after (D3L) an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₂.

25D2F plasma concentration curve, with a curve area ratio of 1.45 ($P \leq 0.01$; Figure 4), regardless of the larger area under the D2L plasma concentration curve compared with the D2F plasma concentration curve (Figure 1). The area under the 25D3L plasma concentration curve was smaller than the area under the 25D3F plasma concentration curve ($P \leq 0.01$; Figure 5), the ratio between the curve areas being 1.63.

The curve area ratios between 25D2F (Figure 4) and D2F (Figure 1; 1.57; $P \leq 0.001$) and between 25D3F (Figure 5) and D3F (Figure 2; 1.24; $P = 0.09$) plasma concentration curves (i.e., when D₂ and D₃ were given as first treatments) were >1.0 , whereas curve area ratios between 25D2L (Figure 4) and D2L (Figure 1; 0.88; $P = 0.3$) and between 25D3L (Figure 5) and D3L (Figure 2; 0.83; $P = 0.3$) plasma concentration curves (i.e., when D₂ and D₃ were given as last treatments) were <1.0 ($P = 0.3$).

DISCUSSION

When comparing areas under the plasma concentration curves, the amount of D₂ (Figure 1) and 25(OH)D₂

Figure 3. 25-Hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ concentrations in plasma (ng/mL; $\pm$ SEM) after an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₂ (25D2F) or vitamin D₃ (25D3F), respectively. Initial plasma concentrations are marked as dotted lines.

Figure 4. 25-Hydroxyvitamin D₂ concentration in plasma (ng/mL; $\pm$ SEM) after an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₂ given before (25D2F) or after (25D2L) an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₃.

(Figure 3) found in dairy cows in the present study when D₂ was given as first treatment (D2F) was much less than half of the amount of D₃ (Figure 2) and 25(OH)D₃ (Figure 3) found when D₃ was given as first treatment (D3F). This might be due to competition between D₂ and D₃ for DBP transport proteins in plasma or for the liver-based vitamin D-25-hydroxylase enzyme system, as speculated by Tjellesen et al. (1986), Trang et al. (1998), and Armas et al. (2004), who reported that D₂ treatment in humans caused declining plasma 25(OH) D₃ concentrations.

Hymøller and Jensen (2010b) showed that when 250 mg of D₂ and D₃ were given simultaneously to high-yielding dairy cows, ingested D₂ did not increase the plasma concentrations of 25(OH)D₂ and D₂ as much as ingested D₃ increased the plasma concentrations of 25(OH)D₃ and D₃. Sommerfeldt et al. (1983) found, in 2 groups of bull calves fed a single oral dose of [³H]-labeled D₂ or [³H]-labeled D₃, that D₂ and D₃ were the predominant vitamin D metabolites circulating in plasma 10 to 15 h after the oral dose was given and that plasma concentrations peaked in 24 to 48 h, which

Figure 5. 25-Hydroxyvitamin D₃ concentration in plasma (ng/mL; $\pm$ SEM) after an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₃ given before (25D3F) or after (25D3L) an oral bolus of 250 mg (1.0×10^7 IU) of vitamin D₂.

is supported by findings of the current study and the study by Hymøller and Jensen (2010b). Plasma concentrations of D₃ and its liver-derived metabolites in calves fed labeled D₃ in the study by Sommerfeldt et al. (1983) were at least twice as high as the concentrations of D₂ and its liver-derived metabolites in calves fed labeled D₂.

Plasma concentrations of metabolites related to the inactivation and degradation of vitamin D in D₂-fed calves were between 25 and 50% of the concentration of the similar metabolites in D₃-fed calves (Sommerfeldt et al., 1983), indicating that there was little surplus vitamin D to clear from the body in calves fed D₂. This indicates differences in intestinal uptake of D₂ and D₃ because D₂ taken up from the intestinal tract would give rise to degradation products in plasma if D₂ were present in plasma and unable to bind to DBP as speculated. These findings do not agree with Hollander (1976) and Hollander et al. (1978), who stated that D₂ and D₃ are taken up by passive diffusion with the fat fraction of the feed.

In the present study, an adverse effect of previous supplementation with D₂ on the effect of subsequent supplementation with D₃ was shown. It should be kept in mind that the D2L and D3L treatments were given to cows that had received a D₃ or D₂ bolus, respectively, 2 wk previously; hence, they had a higher initial plasma status at the beginning of the D2L and D3L treatments than at the beginning of the D2F and D3F treatments. In dairy cattle and other animals fed large amounts of roughage, the effect of D₂ on the physiological efficiency of D₃ could be important when assessing D₃ needs for production, because D₂ is produced by fungi naturally occurring on plant material used for roughage (Richardson and Logendra, 1997).

CONCLUSIONS

In conclusion, oral administration of D₃ increased concentrations of D₃ and 25(OH)D₃ in plasma more efficiently than D₂ increased concentrations of D₂ and 25(OH)D₂. The D₃ given after an oral dose of D₂ was less efficient for increasing plasma concentrations of 25(OH)D₃ compared with D₃ given without previous D₂ administration, whereas the plasma concentrations of D₃ were not affected by previous D₂ administration. The same occurred for plasma concentrations of D₂

metabolites if D₂ was given after D₃, but the effect was much less pronounced.

ACKNOWLEDGMENTS

The Faculty of Agricultural Sciences of Aarhus University (Tjele, Denmark) is acknowledged for financially supporting the presented research.

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